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Mutations in the *KRAS2* oncogene during progressive stages of human colon carcinoma

(tumor progression/polymerase chain reaction/flow cytometry/cell sorting)

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ABSTRACT **A series of colon carcinomas, adenomas, and adjacent tissues were analyzed for ploidy alterations and mutations in *KRAS2*. To increase the sensitivity for identifying mutations, we used histological enrichment, cell sorting, and DNA amplification by the polymerase-catalyzed chain reaction followed by direct DNA sequence analysis. Of the 40 carcinomas analyzed, 27 contained aneuploid cells and 26 contained mutations at the first position of codon 12 of *KRAS2*. Of the 12 adenomas studied, 4 contained aneuploid cells and 9 contained the same mutation at codon 12. In both adenomas and carcinomas, mutations were identified in both diploid and aneuploid cells. In some cases, regions of histologically benign mucosa adjacent to the carcinoma contained mutations. These combined results suggest that mutations in *KRAS2* occur early in the development of human colon carcinoma, before change in ploidy, and that these mutations exist in diploid cells from which an aneuploid subpopulation arises. Furthermore, mutations may exist in histologically normal mucosa in regions adjacent to carcinoma, suggesting that a field of genetically abnormal mucosa may surround these tumors.**

Neoplastic progression is a multistep process characterized by genetic instability, loss of proliferative control, and clonal evolution (1–5). Mutations in cellular *ras* genes have been strongly implicated in this process (6). In humans, mutations within three *ras* genes, *HRAS1*, *KRAS2*, and *NRAS*, have been reported in diverse tumors including myeloid leukemias, colon carcinomas, and pancreatic, thyroid, and renal cell carcinomas (7–14). With colon carcinomas, investigators have further observed that *ras* gene mutations occur in villous adenomas adjacent to regions of carcinoma. These results suggest that *ras* mutations may not only be an early event in the temporal development of colon cancer but also contribute directly to the malignant phenotype (7–9).

Efforts to elucidate the relationship between the occurrence of *ras* mutations and alterations in the behavior of colon tumors have used techniques of DNA amplification and oligonucleotide hybridization or RNase A mismatch-cleavage analysis on fresh or frozen human tissues (7–9). With these techniques ≈40% of colon carcinomas and adenomas have been shown to contain *ras* mutations, the most frequent being a G → A transition at the second position of codon 12 or 13, resulting in a change from glycine to aspartic acid.

However, these methods have several limitations. Quantitation of the frequency and nucleotide position of the mutation is difficult because most tumors consist of a heterogeneous population including epithelial cells, inflammatory cells, fibroblasts, and smooth muscle cells. The presence of nonneoplastic cells in these tumors can produce ambiguities in both the qualitative and the quantitative interpretation of results. The specific assignment of mutations to tumor cells requires purification of the tumor cell population. Moreover, oligonucleotide hybridization techniques have been important for establishing the presence of mutations but do not unambiguously identify the site and nature of the mutation. Also, analyses using fresh tumors do not allow one to retrospectively follow the temporal relationship between the development of adenomas, carcinomas, and mutations in individual patients. Studies with paraffin-embedded archival tissues would allow one to retrospectively reconstruct the role of *ras* mutations in tumor progression.

To increase the sensitivity of detection of mutations within tumor cells, and to specify the site and nature of the mutation, we have used a combination of histological enrichment, cell sorting, polymerase-catalyzed chain reaction (PCR), and direct sequencing on paraffin archival tissues to reconstruct the role of *ras* mutations in colon tumorigenesis.

In contrast to previous reports, we observed that the majority of human colon carcinomas (65%) and adenomas (75%) contain mutations in *KRAS2*. DNA sequence analysis indicated that mutations occur almost exclusively in the first position of codon 12. We observed mutations in both diploid and aneuploid subpopulations of carcinomas, suggesting that mutations precede alterations in ploidy. Mutations were detected not only in benign adenomas but also occasionally in histologically normal mucosa adjacent to regions of carcinoma. Thus, our results suggest that mutations may occur far earlier in tumorigenesis than previously reported and that a field of genetically abnormal mucosa may exist from which such carcinomas arise.

MATERIALS AND METHODS

Histology. *Paraffin-embedded material.* Paraffin archival blocks from formalin-fixed tissue of 40 cases of adenocarcinoma of the colon and 12 cases of tubulovillous adenoma without carcinoma were used as source material. Blocks were selected from patients who had no history of chemotherapy or prior malignancy. In each case, a histologic section was examined microscopically and the paraffin block was cut to separate carcinoma from the adjacent histologically normal mucosa, the underlying smooth muscle, or the adjacent villous adenomatous regions. Each portion was reembedded and examined histologically, to ensure separation of neoplastic from normal tissue, and was sectioned sequentially as follows: a 6-μm section for histologic analysis, one to three 50-μm sections for DNA extraction, two 50-μm sections for flow cytometric analysis, and a 6-μm section for histologic analysis. This procedure allowed serial histologic confirmation of the material utilized in the flow cytometric and DNA sequence studies.

Fresh material. Fresh specimens were obtained from surgery and either directly processed or cryopreserved in

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Abbreviation: PCR, polymerase-catalyzed chain reaction.

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Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% calf serum and 10% dimethyl sulfoxide.

Flow Cytometry. For analysis of fresh specimens, nuclei were isolated and stained with 4',6-diamidino-2-phenylindole (DAPI; 10 μg/ml) by the single-step detergent technique (15). For analysis of paraffin-embedded material, the nuclei were extracted by the procedure of Hedley *et al.* (16). Flow cytometry was performed with an ICP-22 (Ortho Diagnostic Systems), and DNA-content histograms were analyzed as described (17).

Cell Sorting. Aneuploid populations identified by flow cytometry were sorted on an Ortho system 50/2150 cell sorter (Ortho Diagnostic Systems) by using the UV lines from an argon ion laser (351–364 nm). Analysis was performed, and subsets of cells of interest (e.g., diploid vs. aneuploid) were identified within regions used for sorting, as defined with the 2150 computer, and sorted at a rate of up to 1500 cells per sec, with purity >97%.

DNA Extraction. Genomic DNA was extracted from fresh tissue samples by standard methods (18). DNA from paraffin-embedded material was extracted by the method of Goelz *et al.* (19).

PCR. Genomic DNA (0.1–1 μg) was incubated in a total volume of 100 μl of solution containing 50 mM KCl; 10 mM Tris·HCl (pH 8.3); 10 mM $MgCl_2$; 0.1% gelatin; 750 μM each dATP, dGTP, dCTP, and dTTP; 500 ng of HPLC-purified oligonucleotide primers (Operon Technologies) and 2.5 units of *Thermus aquaticus* (*Taq*) DNA polymerase (Perkin-Elmer Cetus). Templates were denatured for 2 min at 95°C, followed by 30–50 cycles of PCR with incubations of 2 min at 43°C (annealing), 3 min at 72°C (polymerization), and 1 min at 94°C (denaturation) (20–22).

The oligodeoxynucleotide primers had the following sequences: 5' PCR primer, 5'-TAAGGCCTGCTGAAAATGACTGAAT-3'; 3' PCR primer (complementary strand), 5'-CTCTATTGTTGGATCATATTCGTC-3'; 5' sequencing primer, 5'-ACTGAATATAAACTTGTGGTAGTT-3'; 3' sequencing primer (complementary strand), 5'-ATTCTGAATTAGCTGTATCGT-3'.

The PCR primers flank a 77-base-pair (bp) sequence that includes codons 4–29 of *KRAS2*. The complementary-strand sequencing primer (3' sequencing primer) provides sequencing information on codons 4–17 of the PCR product, whereas the 5' sequencing primer provides information on codons 10–29.

Sequencing. Products of PCR were extracted with phenol/chloroform (1:1) and centrifuged through Centricon C30 (Amicon) filters. One-fourth of the PCR reaction mixture was then sequenced by a modification of the dideoxy chain-termination method of Sanger *et al.* (23) using 100 pmol of sequencing primer (end-labeled with [γ-^{32}P]ATP and polynucleotide kinase) and 0.5 unit of the Klenow fragment of *Escherichia coli* DNA polymerase I as described (24). When the modified T7 DNA polymerase (Sequenase) was used, sequencing conditions were as recommended by the manufacturer (Sequenase kit, United States Biochemical).

RESULTS

Mutations in the *KRAS2* gene were previously demonstrated in ≈40% of colorectal carcinomas and adenomas by PCR and oligonucleotide hybridization or RNase A mismatch-cleavage analysis (7–9). To maximize detection of mutations in *KRAS2* in human tumors and to study the localization of these mutations within subpopulations of tumor cells, we used a combination of histologic enrichment, flow cytometry, and cell sorting to purify subpopulations of neoplastic cells from stromal, epithelial, or inflammatory cells. DNA extracted from nuclei of these cells was amplified by PCR using primers specific for *KRAS2* and was sequenced by the dideoxy chain-termination reaction.

For each paraffin block, the carcinoma was manually excised from regions containing residual adenoma, histologically normal mucosa, or underlying smooth muscle and reembedded in separated paraffin blocks prior to sectioning for DNA extraction. A 6-μm section was then taken for histologic confirmation, followed by serial 50-μm sections for flow cytometric and PCR analysis. Representative results from the sequencing autoradiogram from PCR-amplified DNA taken after histologic enrichment is shown in Fig. 1 (Table 1, case C-1). In this case, a mutation was detected in the carcinoma and in the adjacent adenoma but not in the adjacent histologically normal mucosa. The G → A substitution at the first position of the coding strand of codon 12 results in an amino acid change from glycine to serine, which has been shown to be an activating mutation for c-Ki-*ras* genes (6–9). The location of this mutation was confirmed by also sequencing the opposite strand. In both the carcinoma and the adenoma, a small amount of the normal allele was also detected at this position.

A total of 40 cases of colon adenocarcinoma were then analyzed for DNA content and *KRAS2* mutations. Of the 40 carcinomas, 27 (68 ± 7% standard error) contained cells with an aneuploid DNA content, which comprised 6–82% of the population of cells that had been histologically enriched for carcinoma (Table 1). Sixty-five percent (26/40 = 65 ± 8%) of these tumors contained mutations in codon 12 of *KRAS2*, all but one were G → A substitutions at the first base-pair position; case C-32 exhibited a G → T substitution at the same position. Statistically significant differences were not observed between the percentage of diploid or aneuploid neoplasms with mutations (9/13 = 69 ± 13% and 17/27 = 63 ± 9%, respectively). A higher percentage of stage C lesions was observed to contain aneuploid subpopulations than earlier stages (17/21 = 81 ± 9% for stage C, 10/19 = 53 ± 11% for stages A and B combined), but this was not statistically significant.

Diploid and aneuploid subpopulations from carcinomas from nine patients were sorted and independently subjected to PCR and sequencing analysis. As expected, the three cases

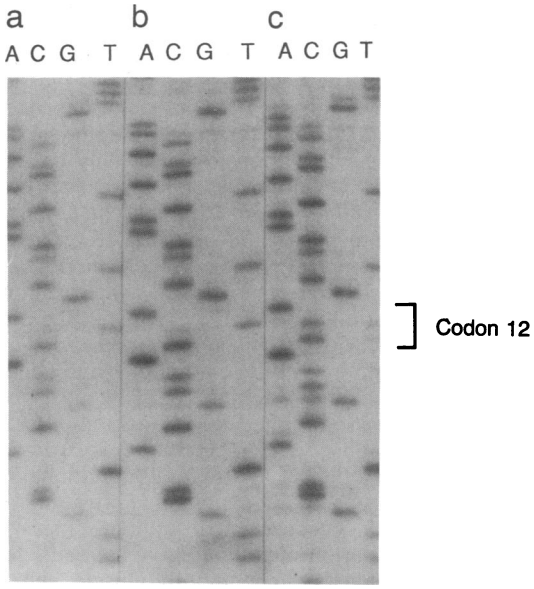

FIG. 1. Sequencing autoradiogram of the complementary strand of *KRAS2* from PCR-amplified DNA extracted from carcinoma (*a*), adenoma (*b*), and adjacent histologically normal mucosa (*c*). The carcinoma and adenoma contain a mutation in codon 12 (ACT) which is absent in the normal mucosa (ACC). This represents a G → A transition at the first position of codon 12 in the coding strand of *KRAS2*.

Table 1. Cumulative data for colon carcinomas

Case	Age, yr (sex)	Stage	Ploidy	% aneuploid cells	*KRAS2* codon 12
C-1	61 (F)	A	2.48	58	(A/G)GT
C-2	58 (F)	A	2.33	19	(A/G)GT
C-3	76 (F)	A	4.0	65	GGT
C-4	75 (F)	A	2.0	0	(A/G)GT
C-5	81 (M)	A	2.0	0	(A/G)GT
C-6	86 (F)	B1	2.0	0	AGT
C-7	82 (M)	B1	4.7	16	(A/G)GT
C-8	72 (F)	B1	3.2	6	(A/G)GT
C-9	75 (M)	B1	2.0	0	(A/G)GT
C-10	68 (F)	B1	2.8	45	GGT
C-11	70 (F)	B1	2.0	0	(A/G)GT
C-12	84 (F)	B2	2.0	0	(A/G)GT
C-13	70 (M)	B2	2.0	0	AGT
C-14	79 (F)	B2	2.0	0	AGT
C-15	66 (M)	B2	3.96	45	(A/G)GT
C-16	78 (F)	B2	2.0	0	GGT
C-17	69 (F)	B2	2.54	40	AGT
C-18	77 (M)	B2	2.74	45	GGT
C-19	89 (F)	B2	3.1	82	(A/G)GT
C-20	74 (M)	C1	2.0	0	GGT
C-21	62 (M)	C1	3.6	30	GGT
C-22	72 (M)	C2	4.0	23	GGT
C-23	88 (F)	C2	2.0	0	AGT
C-24	71 (F)	C2	2.94	14.7	(A/G)GT
C-25	72 (F)	C2	4.0	17	(A/G)GT
C-26	77 (F)	C2	3.6	38	(A/G)GT
C-27	66 (M)	C2	4.52, 2.3	32	AGT
C-28	75 (F)	C2	4.0	11	AGT
C-29	73 (F)	C2	2.5	65	AGT
C-30	71 (F)	C2	3.1	58	AGT
C-31	69 (F)	C2	4.16	68	GGT
C-32	77 (F)	C2	2.7	47	(T/G)GT
C-33	78 (F)	C2	2.6	12	GGT
C-34	72 (F)	C2	3.1	30	GGT
C-35	74 (F)	C2	3.8	31	(A/G)GT
C-36	68 (M)	C2	2.0	0	GGT
C-37	74 (F)	C2	3.1	30	GGT
C-38	68 (F)	C2	2.9	67	GGT
C-39	72 (F)	C2	2.0	0	GGT
C-40	75 (M)	C2	4.2	69	(A/G)GT

Percentage aneuploid carcinomas, 67.5 ± 7% (27/40); percentage of carcinomas with mutations, 65 ± 8% (26/40); percentage of aneuploid carcinomas with mutations, 63 ± 9% (17/27); percentage of diploid carcinomas with mutations, 69 ± 13% (9/13). Table shows a retrospective analysis of stage (Astler–Coller modification of Duke's staging system for colon carcinoma; see ref. 37), DNA content, the percentage of total aneuploid cells present in the sample, and sequence of codon 12 of *KRAS2*. All aneuploid cases also contained a diploid subpopulation; diploid cases are shown as containing a ploidy of 2.0. The percentage of aneuploid cells was calculated by flow cytometric analysis. The complementary-strand sequencing primer was used to sequence the PCR-amplified product of *KRAS2*; cases exhibiting mutations were confirmed by additional sequencing with the coding-strand sequencing primer.

that were negative for mutations prior to sorting remained without evidence of mutations when the aneuploid and diploid subpopulations were analyzed separately. The six cases that exhibited mutations prior to sorting, however, showed sequencing evidence for mutations at the first position of codon 12 in both the diploid and aneuploid subpopulations after sorting (36). In all cases, the presence and position of the mutation were confirmed by sequencing the opposite strand.

We then analyzed 12 tubular adenomas that exhibited no histologic evidence of dysplasia or malignancy (Table 2). In contrast to carcinomas, only 4 of 12 (33 ± 14%) contained an aneuploid subpopulation; in each case the DNA content was near diploid (range 2.23–2.3) and the aneuploid populations could not be cleanly separated from the diploid cells by sorting. Seventy-five percent (9/12 = 75 ± 13%) of the adenomas, however, contained a mutated *KRAS2* gene, and all mutations were located at the first position of codon 12.

Table 2. DNA content and *KRAS2* mutations in adenomas

Case	Age, yr (sex)	Ploidy	% aneuploid cells	*KRAS2* codon 12	*Mae* I digest
A-1	53 (M)	2.23	51	(A/G)GT	+
A-2	75 (M)	2.0	0	(A/G)GT	−
A-3	74 (F)	2.0	0	(A/G)GT	+
A-4	72 (F)	2.3	60	(A/G)GT	+
A-5	65 (M)	2.0	0	AGT	+
A-6	65 (M)	2.0	0	(A/G)GT	+
A-7	74 (F)	2.0	0	(A/G)GT	+
A-8	75 (M)	2.25	40	GGT	−
A-9	61 (F)	2.3	56	AGT	+
A-10	44 (M)	2.0	0	GGT	−
A-11	74 (F)	2.0	0	(A/G)GT	+
A-12	69 (F)	2.0	0	GGT	−

Percentage of aneuploid adenomas, 33 ± 14% (4/12); percentage of adenomas with mutations, 75 ± 13% (9/12). Shown are ploidy, percentage of aneuploid cells within the sample, sequence of codon 12 in PCR-amplified *KRAS2*, and results of *Mae* I restriction endonuclease digestion of the PCR product. For *Mae* I-digested DNA, + indicates the presence of a 79-bp band confirming the presence of a G → A transition at the first position of codon 12.

The position of the mutation in these adenomas was then confirmed by restriction endonuclease digestion. *Mae* I recognizes the sequence CTAG, which is present in alleles that contain a G → A transition in the first position of codon 12 (25). Digestion of the 126-bp PCR products of *KRAS2* with *Mae* I produces a 79-bp band only when there is a G → A transition at the first base pair in codon 12; both normal alleles and mutations at other locations do not produce sequences recognized by the endonuclease (Fig. 2). All nine samples that contained a mutated sequence by sequencing analysis were digested by *Mae* I to produce a 79-bp band.

Additional controls demonstrated that fixation or paraffin-embedding procedures did not contribute to the production of mutations in *KRAS2*. Mutations were not seen in fresh or fixed samples of four placentas, lymphocytes from two patients, lymph nodes from two patients, two lung specimens, two breast carcinomas, diploid fibroblast cells, a simian virus 40 (SV40)-transformed Werner syndrome cell line, and the T24 bladder carcinoma cell line after PCR amplification of the DNA. The known G → A transition at the first base pair of codon 12 was identified in a plasmid containing the *ras* gene from Kirsten murine sarcoma virus (26); a G → A transition at the second base pair of codon 12 was seen in a rat Ki-*ras* plasmid containing this mutation and was absent from a nonmutated rat c-Ki-*ras* plasmid (27).

In two carcinoma cases, mutations were observed in the mucosa immediately adjacent to the carcinoma. In both of these cases, the contiguous mucosa was excised away from the carcinoma and reembedded in paraffin, and alternating sections were cut for histologic analysis, flow cytometry, and PCR analysis. Mutations were seen in the histologically normal mucosa despite the absence of microscopic evidence for dysplastic or neoplastic cells or flow cytometric evidence of aneuploidy within the specimen (Fig. 3; case C-17). To rule out the possibility that these mutations were present constitutionally in these patients, 10 cases of colon carcinoma with adjacent normal mucosa, underlying smooth muscle, and normal mucosa and smooth muscle from the proximal or distal surgical margins were analyzed for mutations in *KRAS2*, both in freshly obtained tissues and after fixation and embedding. Mutations were observed in the same two

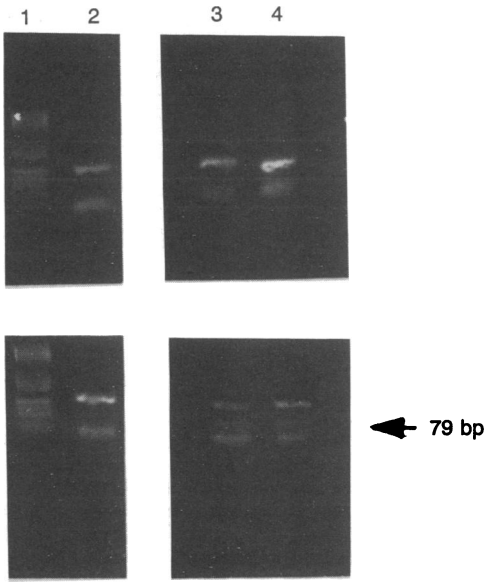

FIG. 2. NuSieve (4%; FMC) agarose gel of PCR-amplified *KRAS2* sequence before (*Upper*) and after (*Lower*) digestion with *Mae* I restriction endonuclease for 1 hr at 45°C in buffers supplied by the manufacturer (Boehringer Mannheim Biochemicals). A 79-bp band is seen in two cases (lanes 3 and 4) that showed evidence for a G → A transition in the first position of codon 12. This band is absent from the *Mae* I-digested DNA from the case (lane 2) that showed no evidence for mutation. Lane 1 contained *Hae* III fragments of plasmid pBR322 as molecular size standards.

cases of colon carcinomas before and after fixation; fresh carcinomas without mutations were not observed to contain mutations after fixation, and mutations were not observed in smooth muscle or mucosa from the proximal or distal surgical margins in the same cases that contained mutations in the carcinoma.

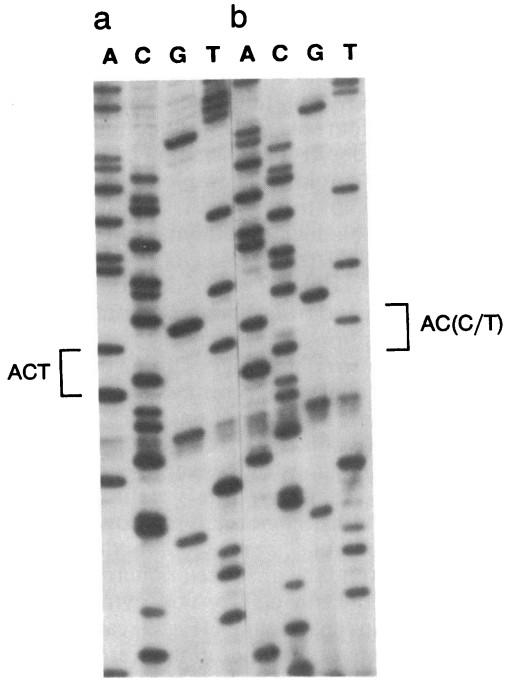

FIG. 3. Sequencing autoradiogram of complementary strand of PCR-amplified *KRAS2* in carcinoma (*a*) and adjacent histologically normal mucosa (*b*). Both the carcinoma and adjacent mucosa contain the sequence ACT at codon 12, which represents a G → A transition at the first position in the coding strand.

DISCUSSION

We have used a combination of histologic enrichment, cell sorting, *in vitro* DNA amplification, and direct sequencing to retrospectively analyze the histologic distribution and spectrum of mutations in *KRAS2* in colon carcinomas and adenomas. In agreement with other investigators, we observed mutations in codon 12 of *KRAS2* in a large percentage of carcinomas.

In contrast to previous investigators, however, we observed a higher frequency of mutations in both carcinomas and adenomas (65% and 75%, respectively). Moreover, we detected mutations in both diploid and aneuploid adenomas and carcinomas, as well as in both diploid and aneuploid subpopulations of individual carcinomas. Although both adenomas and carcinomas have been shown to be monoclonal populations of cells, the majority of adenomas are diploid in DNA content, whereas carcinomas are largely aneuploid (4, 28–31). Our combined flow cytometric and mutational analyses suggest not only that mutations in *KRAS2* occur early in the progression from adenoma to carcinoma but also that these mutations may precede recognizable alterations in ploidy as detected by flow cytometry. The diploid population of these neoplasms may therefore be substantially composed of a monoclonal population of neoplastic cells, from which the aneuploid subpopulation arises.

Of the 35 mutations we identified within codons 4–37 of *KRAS2* in human carcinomas and adenomas, 34 occurred at the first position of codon 12. This is in contrast to previous studies using oligonucleotide hybridization or RNase mismatch-cleavage analysis, which showed a wider spectrum of activating mutations within codon 12 or 13 (7–9). Irrespective of the location of the mutation, however, all studies have shown a high incidence of *ras* mutations in colorectal carcinomas. This difference in precise localization of the mutation could be due to variations in the patient populations but could also be due to the result of a higher precision in localization by nucleotide sequencing.

Direct sequencing offers several advantages over hybridization procedures. The position and nature of the mutations and identity of the gene can be unambiguously visualized within the sequencing ladder. Although sequencing autoradiograms may not be sufficiently sensitive to detect a small subpopulation of alternative mutations at each base-pair site, the predominant population can be clearly recognized. Moreover, we have performed mixing experiments between PCR products containing mutated and nonmutated sequences and have been able to recognize a minority band that comprises 10% of the sample (data not shown).

We have confirmed the location of the mutation by sequencing both the coding and complementary strands of the PCR product and by restriction endoclease digestion with an enzyme (*Mae* I) that recognizes a sequence produced by a G → A transition at the first base pair of codon 12.

In addition, we have amplified and sequenced *KRAS2* DNA from a variety of nonneoplastic and neoplastic tissues (both fresh and after fixation and paraffin embedding), as well as plasmids with known *ras* mutations, and have not detected spurious mutations other than those that were known to be present in v-*ras* and in a rat plasmid with a G → A transition in the second position of codon 12.

In a small number of cases, mutations were observed within *KRAS2* in histologically benign mucosa immediately adjacent to regions of carcinoma. Although we cannot rule out the possibility that contaminating cell debris or neoplastic cells may have contributed to the PCR, absence of aneuploidy as detected by flow cytometry suggests that the contaminating population was <4% of the total cells, which would not be detected within the sequencing autoradiogram. Moreover, histologic sections taken immediately before and

after sections taken for PCR revealed no microscopic evidence for dysplastic or neoplastic cells. Examination of other tissues from the same patients revealed that these mutations were not constitutional and, indeed, were absent even from the surgical mucosal margins of the colectomy specimens. Flow cytometric studies previously identified topographical regions of increased proliferative activity in normal mucosa immediately surrounding carcinomas (32–35); our results suggest that genetic alterations may also exist in these regions. Moreover, such patients may harbor a field of genetically abnormal mucosa, which may predispose to the formation of further neoplasms. It is possible that the early recognition of *ras* mutations may provide a clinical marker for the identification of patients at higher risk for the development or recurrence of colonic neoplasia and ultimately modify the surgical treatment of these patients.

Previous studies on nonsorted tumor cell populations have reported absence of *KRAS2* mutations in 60% of colon carcinomas. This could have been the result of a lack of sensitivity in the detection of this mutation in a mixed population of cells. Our finding that not all sorted aneuploid tumors contain a mutation in *KRAS2* at codon 12 or 13 demonstrates that this mutation is not invariably associated with human colon cancer. It is possible that the tumors in which we were unable to detect mutations in codon 12 or 13 might contain mutations in other codons of *KRAS2* (e.g., codon 61) or in other *ras* genes (*NRAS* or *HRAS1*). Alternatively, a subset of colon cancers may develop by means that do not involve mutations in *ras* genes. It is, however, also conceivable that mutations in the *ras* gene are a required early event but are lost due to deletion and clonal evolution during tumor progression or by reversion of a single base mutation. The latter hypothesis predicts that this position is hypermutable—a prediction that could be verified by *in vitro* studies on the fidelity of DNA synthesis.

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